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アルカリプロテアーゼ産生を増強したバシラス属細菌作製のための変異原処理至適条件の検討

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Optimization of Mutation Conditions of *Bacillus* sp. to Increase the Yield of Alkaline Protease

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Summary

Alkaline protease is an important group of industrial enzymes whose applications are mainly in laundry detergent and leather processing. Use of enzymes in leather processing instead of chemicals prevents environmental pollution. *Bacillus* sp. is one of the best sources of alkaline protease. The purpose of this study was to augment the alkaline protease production from *Bacillus* sp. MA6 through hydroxylamine mutagenesis. Four treatment periods (30, 60, 90 and 120 min) of three concentrations (100, 200 and 300 μ g/ml) and two testing models of mutant screening (casein hydrolysis and enzyme production) were performed for optimization of mutation conditions.

Higher concentration of hydroxylamine become lethal to *Bacillus sp*. Colony size and color was also changed to large and yellow after mutation. Zone diameters of casein hydrolysis in mutants were 12-18 mm, quite larger than wild strain (8-10 mm). Statistical analysis showed the average values of zone of casein hydrolysis were bigger at 60 min treatment period of 100 μg/ml than any other mutation conditions. In mutation frequency analysis, relative frequency of positive mutants was high (60.5%) in 100 μ g/ml as compared with other concentrations. 60 min treatment period also showed higher average enzyme activity than the other treatment periods. Therefore, 60 min treatment period and 100 μg/ml hydroxylamine supported the better mutation. The mutant M609 was found to be a potent one with 53.9 U/ml enzyme activity, 8-fold higher than that of wild strain (6.5 U/ml). The enzyme production is directly associated with soluble protein concentration and biomass production.

Keywords：*Alkaline Protease, Bacillus sp, Mutation, Hydroxylamine*

INTRODUCTION

Industrial enzymes and their production are of commercially, analytically and clinically important. Proteases, a group of key industrial enzymes, can be produced from animals, plants and microorganisms. For numerous technological and economical reasons, microorganisms are the best source of proteases (1). Microbial proteases play an important role in biotechnological process accounting for approximately 59% of the total enzymes used (2). Alkaline proteases secreted by both neutrophilic and alkalophilic *Bacilli* are of interest because they represent a major source

of commercially produced proteolytic enzymes (3). They are mostly produced extracellularly (4) and having a molecular weight ranging from 20 to 30 kDa are stabilized by Ca^{2+} and have characteristically high isoelctric point (5). The applications of the extra cellular alkaline proteases are mainly concentrated in laundry detergents and leather processing (6, 7). The main microbial sources of alkaline protease are *Bacillus subtilis, B. licheniformis, B. amylofaciens* and *B.stearothermophilus* (8).

Leather is one of the major exportable items in Bangladesh. All of 175 tanneries in Bangladesh, which use lime sulphide process for hair depletion from the skin, deserve quality-processing techniques. This process is now clearly recognized to be environmentally objectionable accounting for 100% of the sulphide and over 80% of the suspended solid of the tanneries effluents (9). Sulphide is highly toxic with obnoxious odor, which causes serious health hazard to the tannery workers (10). Use of enzymatic process in tannery instead of chemical process prevents the environment from pollution problems, improves the leather quality and also reduces the cost that involved in chemical importation.

Previous studies revealed that in shake culture the protease production from *Bacillus* MA 6 was almost growth associated and reached its maximum activity to 7.5 U/ml (11). This rate of production is not enough to commercialize these enzymes and it is necessary to improve the strain. Mutation is the prerequisite step for strain improvement. Hydroxylamine was chosen as a mutagen because this chemical provides high frequency of potent mutants. Again hydroxylamine is less carcinogenic and more available than any other potent mutagen and also easy to handle in laboratory. The alkaline protease enzyme is an inducible enzyme. Certain inducers can enhance their production and organisms must have some regulatory systems to control their production. All these control systems are gene-mediated. Although the site of action is not defined but the mutagen causes hydroxylation of various bases (Fig 1) that may help to express high level of production genes in certain strain. The objectives of the present study were summarized as follow: (i) Mutation of *Bacillus* MA 6 strain at three different concentrations (100 μ g/ml, 200 μ g/ml and 300 μ g/ml) of hydroxylamine and four different treatment times (30min, 60min, 90min and 120min). (ii) Selection of mutants through two subsequent models of screening; casein hydrolysis and enzyme activity, and (iii) Development of methodology, which will indicate the best mutagen concentration and the best treatment time.

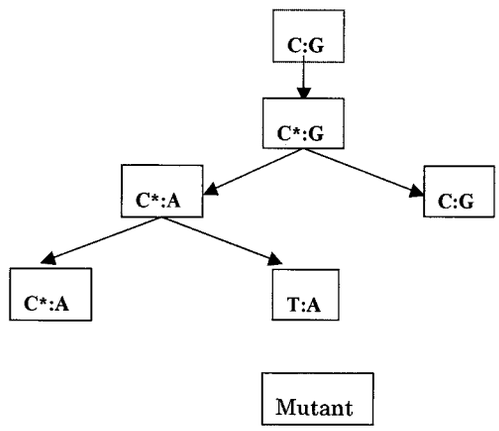

Fig 1: Mutation by hydroxylamine. Hydroxylamine complexes with cytosine forming C*, cytosine (C*) pairs with adenine rather than with guanine by two hydrogen bonds. The C*:A pair will produce a normal C:G pair and a mutant A:T pair during the next round of replication, that is, hydroxylamine induces C:G to T:A transitions.

MATERIALS AND METHODS

Bacterial strain and culture condition. *Bacillus* sp. MA 6, a locally isolated strain, was obtained from the laboratory of the microbiology Department, university of Dhaka for present investigation. The strain was previously used by Azad A.K (11) in his M. Sc. thesis. Nutrient Agar (NA) plates were used for viable count of mutants. Isolation Medium (IM) was used to check the colonies. IM medium is selective for alkalophilic bacteria. Skimmed Milk Agar (SMA) plates were used for the determination of proteolytic activity of the mutants. Alkaline Protease Production Broth (APPB) was used for shake culture production of the alkaline protease enzymes.

Mutagen and mutation. Hydroxylamine was used in this study as a chemical mutagen for mutation. The

culture is treated by hydroxylamine at three different concentrations of 100, 200 and 300 μg/ml at four different treatment times (30, 60, 90 and 120 min). 12 h shake culture (5 ml) was centrifuged at 4000 rpm for 20 min. Pellet was suspended in 4 ml citrate buffer (3.6 ml buffer + 0.4 ml mutagen) and incubated for 30, 60, 90 or 120 min at 37℃. After incubation the cell suspension was centrifuged again at 4000 rpm for 20 min and pellet was resuspended in buffer. Spread plating was done manually to get the isolated colonies.

Microscopic and viable count. Microscopic count was done by Neuber Chamber Counting method before and after mutagen treatment. Viable number of the bacteria was also measured from NA plates by using colony counter before and after mutation.

Measurement of proteolytic activity. Proteolytic activity of the isolated colonies recovered from mutagen treatment was determined on the basis of their ability to produce clear zone of casein hydrolysis on SMA medium. Diameter of the zone was measured in millimeter. The mutants produced greater zone of hydrolysis as compared with that of wild strain were selected for further screening of alkaline protease production.

Alkaline protease production in shake culture. 10ml seed culture was transferred to 90ml of APPB and was allowed to shake in thermostatic orbital shaker for 24 h at 37 ℃ and 120 rpm. After incubation, the samples were taken for centrifugation at 4000 rpm for 20 min and supernatant were used for crude enzyme assay. Protease activity was determined through Azocasein Digest Method described by Kreger and Lockwood (12). Briefly, 400 μl of 1.5% azocasein solution in 0.05M Tris-HCl buffer (pH 8.5) was incubated with 800 μl crude enzyme for 30 min at 40℃ in a water bath with shaking. The reaction was stopped by the addition of 2.8 ml of 5.0% trichloroacetic acid (TCA) for 15 min at 4℃. After centrifugation at 4000 rpm for 20 min, 2 ml supernatant was mixed with 2 ml of 0.5M NaOH and the absorbance was taken at 440 nm. One unit of protease activity was defined as the amount of enzyme that produced an increase in absorbance of 0.01 under the assay conditions.

Estimation of extracellular soluble protein. Soluble protein in the culture supernatant was estimated according to the Bradford method (13). Bovine serum albumin (BSA) of different concentrations was used for standard curve preparation. The amount of soluble protein was determined from standard curve and the concentration was expressed as mg/ml.

Estimation of total dry biomass. The cell mass deposited at bottom of the centrifuge tube was washed, poured on filter paper and dried in oven for overnight at 105℃ and its weight was taken carefully. The amount of total dry biomass was calculated as mg/ml.

RESULTS

Effect of hydroxylamine concentrations on growth and survivability of Bacillus sp MA 6.

Survivability of *Bacillus* sp. are inversely proportional with the increase of mutagen concentration. Viable count is decreased with the increased hydroxylamine concentration from 100 μg/ml to 300 μg/ml and treatment time from 30 min to 120 min (Fig. 2). On the other hand, microscopic count was same before and after treatment. The colony size became larger from 3 mm diameter to 6 mm and the colony color was also changed from white to yellow at 300 μg/ml as compared with lower concentrations.

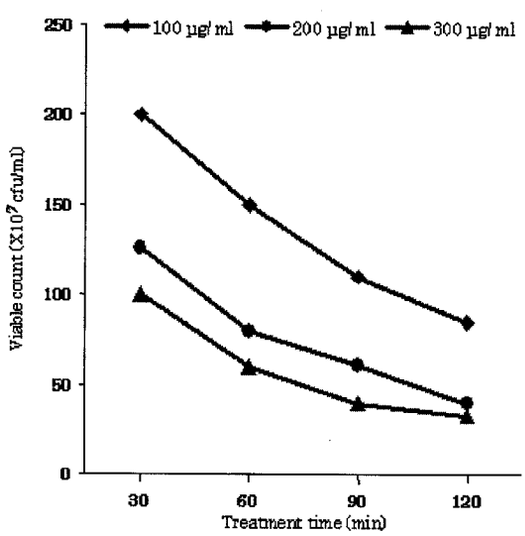

Fig 2: Survivability of *Bacillus* MA 6 strains under different mutation conditions.

Screening of mutants based on casein hydrolysis and enzyme activity

After hydroxylamine treatment at three different concentrations and four different treatment times, mutants were screened on the basis of casein hydrolysis and enzyme production phenomena. For each concentration and each treatment time, only twelve mutants having the higher zone of clearance on casein medium were primarily selected and the selected strains were further screened by their shake culture enzyme production (Data not shown). The zone diameter of casein hydrolysis on SMA at 100, 200 and 300 μ g/ml hydroxylamine concentration were 10 to 18mm, 6 to 15mm and 9 to 15 mm respectively, no marked differences in results were found among these concentrations but increased zone of hydrolysis were noticed as compared with that of wild strain (8-10 mm). The enzyme activity of those selected mutants at different mutagen concentrations were 3.6 to 53.9 U/ml, 2.1 to 19.4 U/ml and 2.8 to 27.3 U/ml respectively. The enzyme activity of wild strain was recorded 6.2 to 7.2 U/ml. The mutant number 9 (named M609) at 60 min treatment time and 100 μ g/ml mutagen concentration was found to give the highest zone of clearance (18 mm) and the highest enzyme activity (53.9 U/ml).

Analysis of mutation conditions for casein hydrolysis

The mean, median and mode values of casein hydrolysis at different hydroxylamine concentrations and treatment times caused by *Bacillus* mutants were shown in table 1. "60 min treatment time" was found to give higher average of zone diameter in each mutagen concentration and "100 μ g/ml" mutagen concentration showed the higher mean value as compared with that of other of 200 and 300 μ g/ml concentration at each treatment time.

Analysis of mutation conditions for enzyme production

The mutants that primarily selected on the basis of casein hydrolysis were further scrutinized for the abilities of enzyme production. The mutants

Table 1 : Statistical analysis of mutation conditions based on casein hydrolysis

Treatment Time (min)	***100 μg/ml****			*200 μg/ml*			*300 μg/ml*		
	*Zone dia (mm)***			*Zone dia (mm)*			*Zone dia (mm)*		
	Mean	*Median*	*Mode*	*Mean*	*Median*	*Mode*	*Mean*	*Median*	*Mode*
30	**13**	13	12	9.6	10	10	12	12	13
60	**14**	**14**	**14**	**13.3**	**13**	**13**	**12.5**	**13**	**13**
90	**12.7**	13	13	12.6	13	13	12.3	12	12
120	**12.5**	13	13	11.9	12	12	11.9	12	12

Zone diameter of casein hydrolysis for wild strain was 8-10 mm

* Mutagen (Hydroxylamine) concentration

** Zone diameter (mm) of casein hydrolysis on SMA

Table 2 : Statistical analysis of mutation conditions based on enzyme activity

Treatment	***100 μg/ml***				*200 μg/ml*				*300 μg/ml*			
time(min)	$f^{(+)ve}$	$f^{(-)ve}$	$r.f^{(+)ve}$	$r.f^{(-)ve}$	$f^{(+)ve}$	$f^{(-)ve}$	$r.f^{(+)ve}$	$r.f^{(-)ve}$	$f^{(+)ve}$	$f^{(-)ve}$	$r.f^{(+)ve}$	$r.f^{(-)ve}$
30	7	5			4	8			7	5		
60	9	3			7	5			5	7		
90	7	5	**60.5%**	39.5%	5	7	41.6%	58.4%	3	9	39.6%	60.4%
120	6	6			4	8			4	8		
	29	19			20	28			19	29		
n	48				48				48			

$f^{(+)ve}$= frequency of positive mutants

$f^{(-)ve}$= frequency of negative mutants

$r.f$= relative frequency

demonstrated two times higher enzyme activity than that of wild strain was termed as "Positive mutants" and the rest were "Negative mutants". The relative frequency (r.f.) of positive mutants were 60.5%, 41.6% and 39.6% at 100, 200 and 300 μg/ml mutagen concentration respectively among which 100 μg/ml of concentration showed more positive mutants than that of other two concentrations (Table 2). The results of average enzyme activity of each treatment time illustrated the highest pick at 60 min treatment time of 100 μg/ml as compared with that other average values (Fig 3).

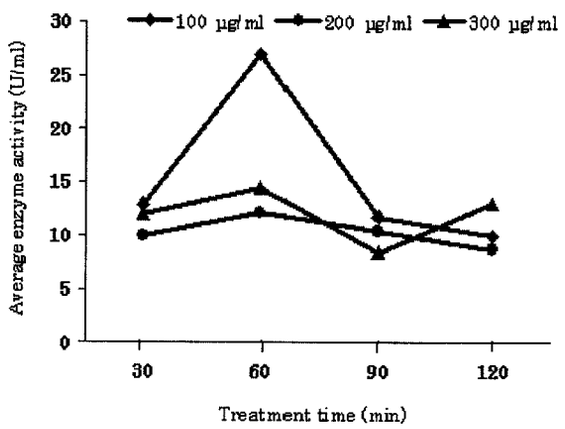

Fig 3: Effect of treatment times on enzyme activity at different hydroxylamine concentrations.

Correlation between casein hydrolysis and enzyme production of Bacillus mutants

Regardless of mutation conditions, some mutants having higher enzyme activity were selected to find out the possible relation with casein hydrolysis. The figure 4 showed that the mutants with higher enzyme activity were able to produce higher zone of clearance on SMA plates and demonstrated a direct correlation with casein hydrolysis.

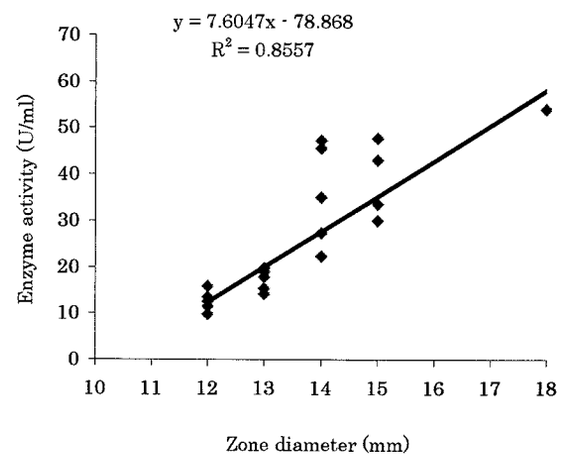

Fig 4: Correlation between casein hydrolysis and enzyme production of *Bacillus* mutants

Effect of hydroxylamine on soluble protein concentration and biomass production

Three mutants of higher enzyme activity from each hydroxylamine concentrations were taken into soluble protein formation and biomass production assay. All of nine mutants gave higher soluble protein concentration and biomass production than that of wild strain (Table 3). The soluble protein concentration and biomass production of wild strains were 0.025mg/ml and 3.24mg/ml respectively. All three mutants under 100 μg/ml of mutagen treatment gave the higher protein concentrations and biomass production than that of mutants treated with 200 and 300 μg/ml of mutagen. The mutant 60 (9), namely *Bacillus* sp M609, was found to give the highest soluble protein concentration as well as biomass production.

Table 3: Efffect of mutagen concentrations on soluble protein concentration and biomass production

Mutagen (μg/ml)	*Mutant No.*	*Protein conc. (mg/ml)*	*Biomass (mg/ml)*
	60(9)	**0.09**	**6.4**
	60(8)	0.089	6
100	90(8)	0.064	5
	60(5)	0.035	3.4
	60(18)	0.07	5
200	90(17)	0.045	4
	30(4)	0.028	3
	30(6)	0.05	4
300	120(9)	0.033	3.6

DISCUSSION

Protease represents one of the three largest groups of industrial enzymes and account for about 60% of the total worldwide sales of enzymes (14). Alkaline protease is an important group of industrial enzymes that occupies a large share of enzyme market. Applications of these enzymes are concentrated in laundry detergent and leather processing. Use of enzymatic process instead of chemical process prevents the environment from pollution problems. Many of the tanneries in Bangladesh, however use commercial enzymes (a mixture of alkaline

protease and lipase) in bating step of leather processing and import about 400 MT of bating powder at a cost of TK 2.5 million annually. More foreign exchanges will be saved if Bangladesh tanneries use enzymes in other steps of leather processing. For both technical and economical reasons, microorganisms are preferable as a source of enzyme rather than plants, animals or even fungi (1). Although fungal proteases are active over wide range of pH (4 to 11) and exhibit broad substrate activity, however, they have a lower reaction rate and heat tolerance capacity than do the bacterial enzymes.

The aim of strain improvement programme is to deregulate the regulatory mechanisms of an organism such that maximum metabolic energy is devoted to a single product, either a particular protein or a product from a metabolic pathway. General approaches of strains improvement are mutation (random mutagenesis) and recombination. Random mutagenesis is based on mutation and screening to improve the strain. Alpha-amylase yields from *Bacillus* subtilis have been improved thousand fold by mutation and screening (15). Recently several recombination approaches are used in strain improvement programme, which include gene cloning, transfection, transduction, conjugation etc (16). The approach of random mutagenesis using hydroxylamine, a mutagenic substance with a higher degree of specificity, was introduced in strain improvement programme in this study. The hydroxylamine usually hydroxylates the cytosine bases with the formation of hydroxylamine-cytosine complex (C*) that pairs with adenine rather than guanine by two hydrogen bonds. The C*:A will produce a normal C:G pair and a mutant A:T pair during next round of replication (Fig 1). Three different hydroxylamine concentrations (100, 200 and 300 μg/ml) and four different treatment time (30, 60, 90 and 120 min) were used in mutation to optimize the mutation condition of *Bacillus* sp MA 6.

Hydroxylamine exerted its effect on growth and survivability of *Bacillus* sp. With the increase of mutagen concentration, viable count was found to decline proportionally that suggested the high concentration of hydroxylamine might be lethal to the organisms. But these increased mutagen concentrations and different treatment times did not affect its microscopic count due to the inabilities of Neuber chamber method to distinguish viable cells from dead one. Growth is also affected by hydroxylamine concentrations. Colony size was increased with rising concentration of mutagen and colony color shifted from white to yellow with increased mutagen concentration.

Two successive assays were performed for screening the mutants, which included (i) casein hydrolysis and (ii) enzyme production. As compared with wild strain, colonies having higher zone of clearance on SMA plates were primarily shorted out from each treatment time of every mutagen concentrations. Casein was used as it was thought to be a very potent inducer for the synthesis of alkaline protease, which produced about 90% of extracellular protease (17). The analysis of casein hydrolysis revealed the highest mean, median and mode value (14 mm) at 60 min treatment period regardless of concentrations used. When mutagen concentrations were compared, higher mean values of casein hydrolysis were found at 100 μg/ml at every time period as compared with 200 and 300 μg/ml concentrations (Table 1). So, **60 min treatment time of 100-μg/ml concentration supported the better mutation** of casein hydrolysis.

The mutants selected from first screening (casein hydrolysis) were subjected to allow for shake culture enzyme production to get potent mutants. Shake flask culture was used because of its low cost of production, process control capabilities and reduced contamination problems (17). Optimum incubation period for maximum protease production were adjusted 24 h based on previous work (11), although incubation period varies from with *Bacillus* sp. such as for *B. licheniformis* the incubation was about 36 h, for *B. stearothermophilus* was 24-36 h and for *B. subtilis* was 48 h (18, 19). APPB (Alkaline Protease Production Broth) was used for enzyme production in which $NaNO_3$ was used as nitrogen source because organic nitrogen reduced the proteolytic enzyme production greatly (80-95%) and nitrate increased by 45% the production of alkaline protease (20). The statistical analysis of enzyme production revealed that the highest percentage (60.5%) of relative frequency of positive mutants, which had the

enzyme activity 2 times more than that of wild strain, were found at 100 μg/ml concentration of mutagen treatment (Table 2) as compared with 200 and 300 μg/ml concentration. The average enzyme activity of each treatment time period suggested the 60 min treatment time of 100 μg/ml mutagen concentration is more favorable than other treatment times (Fig. 2). Taken together the results of casein hydrolysis and enzyme production, 60 min treatment time and 100 μg/ml concentration of hydroxylamine might be the best condition for mutation of *Bacillus* sp.

Out of 108 mutants (12 from each treatment time), the mutant number 9 of 60 min treatment time at 100 μg/ml mutagen concentration, named *Bacillus* M609, was found to give the highest enzyme activity (53.9 U/ml) that is 8-fold higher than that of wild strain. Soluble protein concentration and biomass production of nine selected mutants with higher enzyme activity were found to be associated with enzyme production (Table 3).

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アルカリプロテアーゼ産生を増強したバシラス属細菌作製のための変異原処理至適条件の検討

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要旨：アルカリプロテアーゼは重要な工業用酵素群の一つであり、洗濯用界面活性剤や皮革処理に応用されている。皮革処理に際し、化学薬品に換えて酵素を用いることができれば環境汚染を抑制することができる。バシラス属細菌はアルカリプロテアーゼの最良の原料である。本研究の目的は、ヒドロキシルアミンを用いた突然変異によってバシラスMA 6 株のアルカリプロテアーゼ産生を高めることにある。3 種の濃度（100, 200 and 300 μg/ml）で30, 60, 90あるいは120分間処理し、カゼイン水解の変化を指標に変異株をスクリーニングした後アルカリプロテアーゼ活性を調べ、突然変異作製の至適条件を決めた。高濃度のヒドロキシルアミンは供試菌を死滅させた。変異株は、元株より大きな黄色集落を形成した。カゼイン水解の直径は、元株が 8 -10 mmであったのに対して変異株では12-18 mmに達した。カゼイン水解直径の平均値を算出したところ100 μg/mlで60分処理した場合に有意に大きな値を示した。変異頻度を調べたところ、100 μg/mlの処理によってカゼイン水解性が亢進した変異株の60.5 %においてプロテアーゼ産生の増強が認められ、60分間処理した場合に最も高い酵素活性が観察された。変異株M609の酵素活性は、53.9 U/mlであり元株（6.5 U/ml）に比べ8倍高かった。酵素産生は菌が産生する可溶性タンパク濃度およびバイオマス量と直接相関していた。